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Effect of methylene blue based ultrasound on survival and mitochondrial damage of Leishmania tropica promastigotes

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Abstract
Aim: In photodynamic therapy based on visible light-activated photosensitizers, the tissue penetration of visible light is limited, leading to the need for new treatment approachesSonodynamic therapy (SDT) is a treatment modality derived from photodynamic therapy. SDT is a promising new treatment modality combining sonosensitizer and low-intensity ultrasound. In this study, methylene blue (MB) mediated SDT was used to assess survival and mitochondrial damage in Leishmania tropica (L.tropica) promastigotes.
Material and Methods: L.tropica promastigotes, which were incubated with different concentrations (3.125, 6.25, 12.5, 25 and 50μM) of MB for 1 hour, were exposed to ultrasound (US) at a frequency of 1MHz, 50% duty cycle, 7 min with an intensity of 3 W/cm^2. XTT was used to evaluate cell viability and Giemsa staining was used to determine morphological changes. Mitochondria membrane potential (Δψm) was assessed by flow cytometry with JC-1 staining.
Results: With the combination of increasing concentrations of MB and US, L.tropica promastigote viability was found to be decreased compared to the control and US-control group. Giemsa staining findings showed that MB mediated SDT induced several morphological alterations in L.tropica promastigotes typical of apoptosis. The Δψm collapsed significantly when L.tropica promastigotes were treated by US in the presence of MB.
Discussion: US in the presence of MB markedly damaged mitochondrial structure and function and decreased viability of L.tropica promastigotes. Therefore, MB-mediated SDT might be a potential therapeutic modality for L.tropica promastigotes.

Keywords
Leishmania tropica, Methylene Blue, Mitochondria, Sonodynamic Therapy Survival

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Introduction

Leishmaniasis is a worldwide parasitic disease caused by an intracellular parasite transmitted to humans by the bite of Phlebotomus and Lutzomyia [1]. Until now, no effective drug or vaccine for the treatment of Leishmaniasis has been reported. Pentavalent antimony compounds as a standard drug have many side effects. There are still a lot of problems in the treatment of Leishmaniasis, such as increasing cases of parasite resistance, side effects caused by drugs [2]. Therefore, alternative anti-leishmanial treatments based on physical mechanisms such as light and ultrasound are being investigated.

Photodynamic therapy (PDT) is the combination with light and a photosensitizer, always offers an attractive methodology against Leishmania infections due to its low cost and non-invasiveness [3,4]. If light is replaced with low-intensity ultrasound to activate sensitizers, a novel physics-driven treatment emerges. This treatment depends on the interaction between sensitizer and low-frequency ultrasonic waves to generate reactive oxygen species (ROS), which are highly cytotoxic to induce apoptosis in nearly all microorganisms [5-7]. The sonosensitizer has no cytotoxic effect alone and exhibits toxic effects when interacting with ultrasound [8].

Methylene blue (MB) is a first-generation photosensitizer that has been researched for nearly 80 years [9]. MB provides perfect penetration in the cell membrane with a minor concentration in lysosomes, mitochondria and DNA due to its benzene ring [10]. Also, these photosensitizers are generally amphipathic planar molecules that contain one intrinsic quaternary nitrogen atom. Due to its cationic property, MB binds strongly to Leishmania spp. Photosensitizers, which have cationic properties improve the electrostatic interaction of cationic compounds by interacting better with L.tropica promastigotes because of the membrane surface negative charge [11]. Studies show that MB-mediated PDT is effective in reducing the viability of L. Promastigotes [12,13]. However, the efficacy of MB-mediated Sonodynamic therapy (SDT) is not yet known. This study aimed to examine the effect of MB-mediated SDT on the survival and mitochondria damage of L. tropica promastigotes.

Material and Methods

Parasites: L. tropica promastigotes were maintained in RPMI-1640 medium supplemented with 200 U/ml of penicillin, 0.2 mg/ml of streptomycin, and 10% fetal calf serum (both from Cegrogen Biotech GmbH, Germany) incubated at 26°C.

Experimental protocols for the study

The study contained four groups:

1. ***Control group***: No MB, no ultrasound;
2. ***Methylene blue group***: Treatment with all MB concentrations alone;
3. ***Methylene blue mediated SDT group***: ultrasound in the presence of MB;
4. ***Ultrasound group (US-Group)***: Ultrasound treatment at 1 MHz, with a 50% duty cycle and a 3W/cm^2 power intensity for 7 min.

Preparation of sonosensitizer

MB was used as a sonosensitizer. Phosphate buffer solution (PBS) was used as solvent in stock solutions of MB. Stock 200 µM MB solution was prepared as a 1/2 serial dilution with RPMI 1640 cell culture solution. Parasites were added to the wells as 1x10^7 cells and MB concentrations were determined as 50, 25, 12.5, 6.25 and 3.125 µM.

Sonodynamic therapy experimental procedure

Parasites were incubated with 3.125, 6.25, 12.5, 25 and 50µM of methylene blue for one hour and the samples were centrifuged at 1500 rpm for 5 minutes, and subjected to ultrasound at a frequency of 1 MHz, at a distance of 5 cm at an intensity of 3 W/cm^2 at 50% duty cycle for 7 min in water [14]. After the ultrasound application, fresh medium was added to the samples and incubated at 26˚C for 18 hours.

Analysis of cell viability of promastigotes of L. tropica by the XTT cell proliferation test

The XTT test was performed to determine the cell viability of the evaluated promastigotes. 100 µL of the parasite culture was added to the control group (no sonosensitizer, no ultrasound), the sonosensitizer group (treated with increasing concentrations of methylene blue), the ultrasound group (1 MHz, 3W/cm^2, 50% duty cycle) and the SDT group (treated with methylene blue and 1 MHz, 3W/cm^2, 50% duty cycle), and 50 µL of XTT reagent for all the groups analyzed. All absorbance data were acquired at 450 nm using a multimode ELISA reader.

Morphological Analysis by Giemsa staining

Giemsa staining was performed in all groups slides to evaluate morphological alterations. An aliquot with 20 µL from each group was taken and samples were spread onto slides and then air-dried at room temperature. Parasites were fixed with methanol. The parasites were dyed with Giemsa (1:5) for 15 min. Then the samples were washed in running water and allowed to dry. The samples were analyzed in a light microscope.

Mitochondrial Membrane Potential (Δψm) Assay

BD MitoScreen Δψm Detection Kit (BD Bioscience, USA), was used for the detection of Δm alteration by flow cytometry using JC-1 staining according to the manufacturer's protocol. When the cell is healthy, JC-1 commonly appears as red fluorescence and Δψm value is high.

On the other hand, JC-1 appears as green fluorescence in unhealthy cells and Δψm value is low. After being treated with IC50 concentration of the tested MB, samples were centrifuged at 1500 rpm for 5 minutes, and JC-1 working solution was added and incubated for 15 minutes at 26°C. Promastigotes were then washed twice with JC-1 buffer and ΔΨm was determined using the flow cytometer.

Statistical Analysis

The cytotoxic activity and mitochondrial membrane potential alteration of MB and MB-mediated SDT on L.tropica promastigotes were presented and analyzed using one-way ANOVA analysis of variance followed by Tukey post-hoc test via the SPSS 25.0 program. P- value of <0.05 was considered significant.

Results

The cytotoxic activity of MB and MB-mediated SDT on L. tropica promastigotes was calculated using the viability assay to determine the IC50 value of MB. In this study, cytotoxicity of MB-mediated SDT on L.tropica promastigotes by XTT test was determined as 8.623 ± 1.98%, 51.5 ± 5.1%, 62.9± 3.07%, 76.7± 1.35, 80.8± 1.11%, respectively. No effect of MB alone

on L. tropica promastigotes was determined. The viability of L.tropica promastigotes is over 73% (Figure 1A-B).

After MB (25 μM) and 50% pulsed 7-minute ultrasound at 1 MHz frequency and at 3W/cm2 intensity, L.tropica promastigote viability was found to be statistically decreased compared to the control group ($p<0.001$). The observed IC50 of MB-mediated SDT was 25 μM for parasites. In the group with MB-mediated SDT, parasites were detected to be significantly reduced at all concentrations compared to the control group (Figure 1B)

Morphological analysis of Leishmania tropica promastigotes

The morphological analysis of L.tropica promastigotes revealed that the control groups and the ultrasound group showed no morphological changes, maintaining a fusiform appearance, with a single nucleus, kinetoplast, narrow body, and flagellum (Figure 2A and Figure 2G). Alteration of the fusiform shape was observed at the highest MB concentration (50 ψM) (Figure 2B). There was no change in the morphological features of the parasites, except for 50 ψM concentration in the group to which only the MB group was applied (Figure 2C-F). L. tropica promastigotes exhibited morphological alterations such as round and atypical structures after SDT with high concentrations of MB used in the study. After treatments with high concentrations of MB-mediated SDT, L. tropica promastigotes lost their characteristic morphological features such as fusiform shape, nucleus and flagellum, while typical morphological features were determined at the lowest concentrations of MB-mediated SDT (Figure 2H-L).

Evaluation of mitochondrial membrane potential by JC-1 staining

The percentage of cells in the two populations, P3 and P4, was measured as JC-1 fluorescence, which is shown in Figure 3. P3 represents the mitochondrial resting membrane potential and P4 represents the depolarized mitochondrial membrane potential and cells prone to apoptosis. In the control, US control, and MB groups (Figure 3A-C), red fluorescent signals were detected with a higher cell population (93.2%, 91.6%, 70.4%), while green fluorescent signal was observed approximately nine times stronger in the MB-mediated SDT group (46.4%, Figure 3D) than in the control groups (4.6%, 5.6%). It was determined that the ratio of change in mitochondrial membrane potential decreased from 19.5±2.83 to 0.53±0.37 in L.tropica promastigotes treated with MB- mediated SDT at a dose of IC50 (25μM), compared to the controls (Figure 3E).

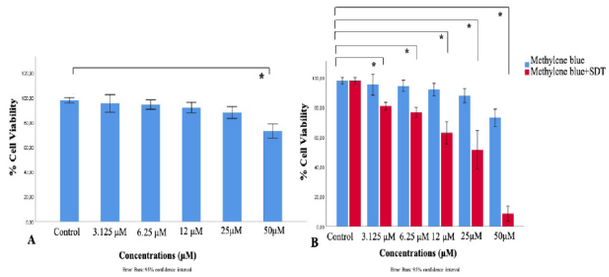

Figure 1. Evaluation of viability after treatment with MB alone and MB-mediated SDT. A. MB groups B. MB-mediated SDT groups. Results are presented as means + SD; n = 3 (*p<0.001, Error bars 95% confidence interval)

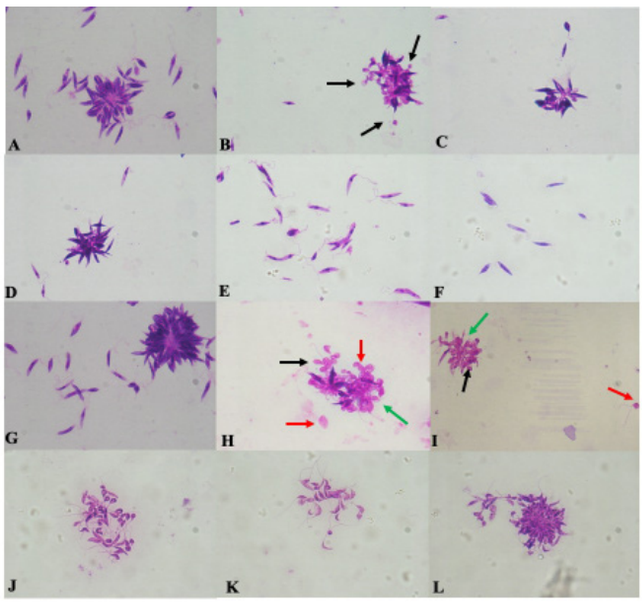

Figure 2. Morphology of L. tropica promastigotes with Giemsa staining for MB and MB- mediated SDT group (x100). A. Control grup B. 50ψM MB, C. 25ψM MB D. 12.5ψM MB E. 6.125ψM MB F. 3.125 ψM MB G. US-Control group H. 50ψM MB+SDT I. 25ψM MB+SDT J. 12.5ψM MB+SDT K. 6.125ψM MB+SDT L. 3.125 ψM MB+SDT. (→) irregular shape, round structures, (→) No nucleus, (→) No flagellum.

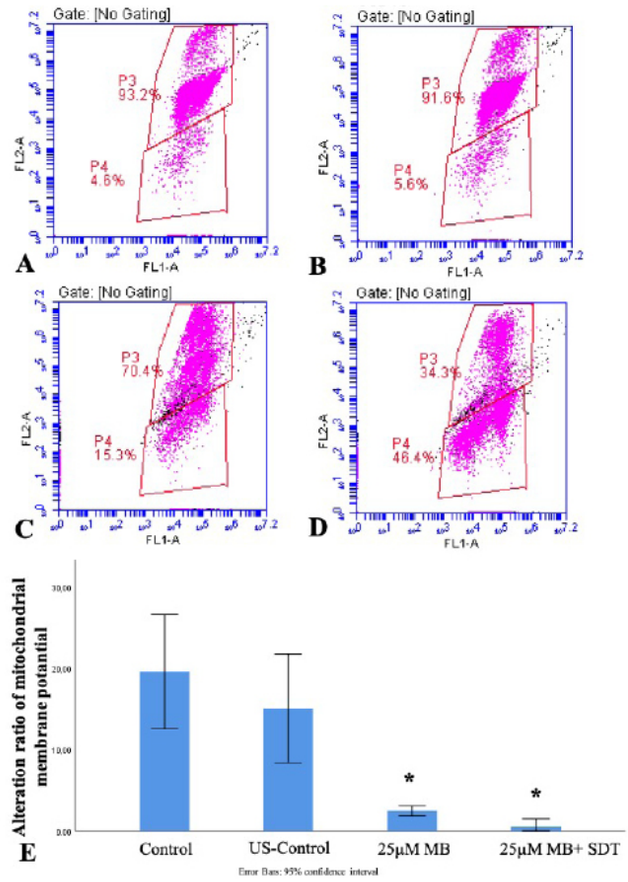

Figure 3. Changes in the ΔΨm of L. tropica after 18 h of incubation with MB , IC50 MB mediated PDT. A. Control B. US-Control C. treatment with 25 μM MB D. treatment with 25 μM MB mediated SDT. The dot plots are representative of three independent experiments E. Bar graphs representing mean alteration ratio of mitochondrial membrane potential. Results are presented as means + SD; n = 3 *p < 0.001.

These results were confirmed by demonstrating the change in mitochondrial membrane potential of apoptotic cell death in L.tropica promastigotes of MB-mediated SDT.

Discussion

Methylene blue is the most examined photosensitizer of the phenothiazine class against Leishmania species due to its high singlet oxygen quantum efficiency of around 0.5, absorption band between 550 and 700 nm, and embodies many features of an ideal photosensitizer [15]. Mitochondria have an important effect on cell apoptosis. They are also known as the "powerhouse of the cell" as they produce most of the cell's source of adenosine triphosphate (ATP) [16,17]. According to various studies, methylene blue is a photosensitizer with a high affinity for mitochondria, due to its hydrophilic/lipophilic nature and the presence of a positive charge. When MB enters the mitochondria, it can be largely retained or accumulated, driven by the mitochondrial membrane potential (ΔΨm). It interacts with nucleic acids, proteins and lipids, modulating their functions [18-20].

Sonodynamic therapy is one of the candidate alternative treatment methods for Leishmaniasis, due to its low cost,, easy of use and non-toxicity. The biological effects of SDT are related to at least one of three different mechanisms, including heat, acoustic cavitation, and mechanical effects. Cavitation was described with microbubbles resulting from pressure changes that occur during the advancement of ultrasound in the tissue fluid [21]. These effects depend on the frequency and intensity of the ultrasound. As a result of the exposure of biological tissues to ultrasound, both structural and functional changes may occur in cells [22].

Basmaciyan et al. suggested the identification of cell death and apoptosis by demonstrating the presence of at least two apoptotic markers in Leishmania. One of the most basic apoptotic markers in Leismania is cell morphology. Regarding morphology, at least two of the five alterations must be present, such as cell shrinkage, cell rolling, preservation of integrity, and changes in the plasma membrane, nuclear fragmentation, and chromatin condensation [23]. This study confirms the findings, as there were also alterations in their morphology with the loss of the shuttle shape and narrow body, as well as the absence of nucleus, kinetoplast and flagella in L. tropica promastigotes. Another apoptotic marker in Leishmania species is mitochondrial depolarization. The loss of ΔΨm is one of the physiological process associated with programmed cell death and necrosis. Current studies assigned that the decrease of Δψm after PDT is the opening of mitochondrial permeability transition pores [24]. However, the mechanism in SDT is not clear. Caliskan-Ozlem et al. applied curcumin based SDT on L. tropica parasites and identified a reduction in mitochondrial activity with a JC-1 probe [14]. In our study, it was observed that the ΔΨm decreased. The decrease of ΔΨm has shown that it may play a key role in the death of L.tropica promastigotes. These findings confirm the involvement of mitochondria in MB-mediated SDT applied cells. Induction of ΔΨm change showed that MB mediated with SDT, also affected the mitochondria of L.tropica promastigotes.

Caliskan-Ozlem et al. incubated L. tropica rose bengal promastigotes in dark for 1 hour and then exposured ultrasound to the promastigotes. This study group determined that the number of promastigotes was lower than in the control group after rose bengal-mediated SDT (1 MHz frequency, at a distance of 5 cm at an intensity of 2W/cm^2). They also confirmed changes in cell morphology by Giemsa staining. This study group stated that atypical cells such as round structures, loss of flagella, nuclei and kinetoplasts were observed after MB-mediated SDT [25]. In our study, it was determined that as the concentration increased after MB-mediated SDT application, cell viability decreased, and there was a significant difference between MB alone and MB-mediated SDT groups in terms of cell viability.

This study has two main limitations. The first limitation is an in vitro study of MB-mediated SDT against L.tropica promastigotes. There are no intracellular amastigotes or experimental animal models. The second limitation is that the reviewed SDT studies differ among themselves, making comparison and rationalization of data uncertain. However, our study also has some strengths. An important contribution will be made to the literature with ultrasound application optimization. Studies of MB-mediated SDT on parasites provide valuable information.

Conclusion

This study confirmed that MB based SDT could remarkably destroy L. tropica promastigotes and highlighted that mitochondria membrane potential depolarization might play an important role in the MB-mediated SDT. Moreover, it showed that MB-mediated SDT could be a potential therapeutic modality that is inexpensive, non-toxic, and non-invasive for treating L. tropica promastigotes.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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